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Francisella tularensis Molecular Typing Using Differential Insertion Sequence Amplification▽

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Tularemia is a potentially fatal disease that is caused by the highly infectious and zoonotic pathogen *Francisella tularensis*. Despite the monomorphic nature of sequenced *F. tularensis* genomes, there is a significant degree of plasticity in the organization of genetic elements. The observed variability in these genomes is due primarily to the transposition of direct repeats and insertion sequence (IS) elements. Since current methods used to genotype *F. tularensis* are time-consuming and require extensive laboratory resources, IS elements were investigated as a means to subtype this organism. The unique spatial location of specific IS elements provided the basis for the development of a differential IS amplification (DISA) assay to detect and distinguish the more virulent *F. tularensis* subsp. *tularensis* (subtypes A.I and A.II) and subsp. *holarctica* (type B) strains from *F. tularensis* subsp. *novicida* and other near neighbors, including *Francisella philomiragia* and *Francisella*-like endosymbionts found in ticks. Amplicon sizes and sequences derived from DISA showed heterogeneity within members of the subtype A.I and A.II isolates but not the type B strains. These differences were due to a 312-bp fragment derived from the IS element IS*Ftu1*. Analysis of wild-type *F. tularensis* isolates by DISA correlated with pulsed-field gel electrophoresis genotyping utilizing two different restriction endonucleases and provided rapid results with minimal sample processing. The applicability of this molecular typing assay for environmental studies was demonstrated by the accurate identification and differentiation of tick-borne *F. tularensis*. The described approach to IS targeting and amplification provides new capability for epidemiological investigations and characterizations of tularemia source outbreaks.

Francisella tularensis is a facultative intracellular bacterium and the causative agent of the zoonotic disease tularemia (10). This Gram-negative microbe is highly infectious, with as few as 10 organisms being capable of causing disease in numerous mammalian species, including humans. Accordingly, *F. tularensis* is considered a potential biological weapon and is classified by the Centers for Disease Control and Prevention (CDC) as a select-agent pathogen (29). The three widely accepted subspecies of *F. tularensis* include *F. tularensis* subsp. *tularensis* (also known as type A), *F. tularensis* subsp. *holarctica* (also known as type B), and *F. tularensis* subsp. *mediasiatica*, whereas classification of the fourth subspecies, *F. tularensis* subsp. *novicida*, remains in contention (13, 14, 20). Differentiation among these subspecies is important due to their variation in virulence and for the tracking of tularemia outbreaks. Although *F. tularensis* subsp. *mediasiatica* was reported to exhibit a moderate degree of virulence in mammals, the geographic distribution in Central Asia and limited accessibility to isolates have restricted the knowledge of this subspecies' pathogenicity (4). *F. tularensis* subsp. *novicida* is generally considered an opportunistic microbe, whereas *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* are capable of causing a severe form of tularemia. Moreover, type A strains (*F. tularensis* subsp. *tularensis*) can potentially cause a life-threatening illness. The virulent type A and type B clades show remarkable geographic associations: *F. tularensis* subsp. *holarctica* is prevalent throughout the Northern Hemisphere, while *F. tularensis* subsp. *tularensis* is restricted primarily to North America (25). Recently, subtypes A.I and A.II of *F. tularensis* subsp. *tularensis* have been correlated with certain regions in North America and differences in virulence, with subtype A.I infection being reported to be more often fatal (18). Therefore, the rapid detection and typing of *F. tularensis* are critical for epidemiological tracking, clinical disease management, and biodefense monitoring.

Numerous molecular studies have been evaluated for the detection and identification of *F. tularensis* from biological reservoirs such as lagomorphs and ticks. However, amplification of the 16S rRNA gene and other targets has produced misleading results due to their cross-reactivity with near neighbors and *Francisella*-like endosymbionts known to exist in ticks (3, 11, 17). In general, examination of the population structure of *F. tularensis* at the genetic level has required more robust and time-consuming approaches, such as pulsed-field gel electrophoresis (PFGE), multilocus variable-number tandem-repeat analysis, and whole-genome detection of single-nucleotide polymorphisms (SNP). The value of these studies has been significant. For example, phylogenetic analyses of repeats and SNP content suggested that *F. tularensis* subsp. *holarctica* originated from North America and was introduced multiple times into Eurasia (15, 33). Furthermore, comparative genome

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hybridization and sequencing were used to demonstrate a unique deletion present within an *F. tularensis* isolate found in an Iberian Peninsula tularemia outbreak (2, 7, 8).

Other genetic markers with the potential for use in molecular typing have been identified. Seventeen contiguous regions were previously identified in *F. tularensis* subsp. *holarctica* that were dispersed in *F. tularensis* subsp. *tularensis* using paired-end sequencing (8). A recent genomic comparison between four *F. tularensis* subsp. *tularensis* isolates (SCHU S4, FSC 198, NE-061598, and FSC 033) confirmed that these subtype A.I strains are clonal, with a high degree of chromosomal synteny (23). Nevertheless, distinctions among these strains could be made based on inversions and rearrangements that were predictably mediated by direct repeats and insertion sequence (IS) elements (23). Additional computational analysis suggested that several of the contiguous regions could be used to differentiate between type A and type B strains. However, since few wild-type isolates have been sequenced, the application of this approach to characterize new foci of tularemia outbreaks was unknown. To address this challenge, a molecular assay was developed using differential insertion sequence amplification (DISA) to characterize wild-type *F. tularensis* isolates, and results were compared to the typing patterns obtained with PFGE using two different restriction endonucleases.

MATERIALS AND METHODS

Bacterial strains. Eighty-eight wild-type and four reference strains of *F. tularensis* were evaluated, as shown in Fig. 2 and 3. The wild-type isolates were obtained from multiple sources, including public health and diagnostic laboratories in the United States. The identities of all *F. tularensis* isolates were confirmed according to the testing protocols utilized by Laboratory Response Network reference laboratories. The reference *F. tularensis* strains included SCHU S4 (subtype A.I), WY96-3418 (subtype A.II), the live vaccine strain (LVS) (type B), and *F. tularensis* subsp. *novicida* (Table 1). Twenty-three non-*Francisella* species representing 21 different species as well as one *Francisella philomiragia* strain were included in an exclusivity panel to assess specificity (Table 1). Select-agent strains were transferred to the University of Nebraska Medical Center, Omaha, NE, according to the requirements of the Select Agent Program as outlined in the Animal and Plant Health Inspection Service/CDC form 2, guidance document for request to transfer select agents and toxins (5). The manipulation of viable culture material was performed by authorized individuals within a biosafety level 3 laboratory certified for select-agent work by the U.S. Department of Health and Human Services, using laboratory biosafety criteria as described by the National Institutes of Health and the CDC (9). All *Francisella* isolates were grown on chocolate agar plates (Remel, Lenexa, KS) and incubated at 37°C with 5% CO_2 for 3 days before processing.

DNA isolation. The extraction of chromosomal DNA from all bacterial species was performed by using CTAB (cetyltrimethyl ammonium bromide) according to standard procedures (34). For the environmental samples, ticks and insects were homogenized in cold phosphate-buffered saline (PBS) within containment by using a Mini Beadbeater-8 instrument (Bio Spec Products, Inc., Bartlesville, OK) according to the manufacturer's recommendations. DNA was then isolated from the arthropod and insect extracts by using the QIAamp DNA blood minikit (Qiagen, Valencia, CA). Pond water and soil samples were subjected to DNA extraction, as was performed for the tick extracts but without homogenization. The DNA from all environmental samples was concentrated following extraction, and approximately 1 ng of DNA was utilized for the PCR assays. If an inhibitor was detected, the DNA was diluted, and 0.1 ng of DNA was used instead. *Dermacentor* and *Amblyomma* ticks were collected from canine or bovine sources as well as by flagging or dragging and were identified to species level according to previously reported keys (16, 35).

PFGE. Agarose-embedded chromosomal DNA for PFGE was prepared and digested with the restriction endonucleases PmeI and BamHI (Fermentas, Inc., Glen Burnie, MD) as previously described (12). Agarose gels (1%, wt/vol) contained *Francisella* DNA digested with PmeI or BamHI and were run for 17.5 h or 16.5 h, respectively, with an initial switch time of 1.8 s and a final switch time of 10.7 s. Migration profiles of the restriction fragments were normalized to SmaI-digested *Staphylococcus aureus* NCTC 8325 by using Bionumerics software (Applied Maths, Inc., Austin, TX). Cluster analysis was performed by using the Dice correlation coefficient and the unweighted-pair group mathematical average (UPGMA) clustering algorithm.

TABLE 1. Reference strains of *Francisella* and non-*Francisella* strains used in this study

Species	Strain designation(s)[c]
Francisella tularensis subsp. *tularensis*[a]	SCHU S4, NR-643[b]
Francisella tularensis subsp. *tularensis*[a]	WY96-3418, NR-644[b]
Francisella tularensis subsp. *holarctica*	ATCC 29684, LVS, NR-14[b]
Francisella tularensis subsp. *novicida*[a]	ATCC 15482, Utah 112, NR-13[b]
Francisella philomiragia	ATCC 25015
Acinetobacter baumannii	Wild type[c]
Bacillus atrophaeus[d]	ATCC 9372
Brucella canis	Wild type[c]
Burkholderia cepacia	ATCC 25608
Corynebacterium pseudodiphtheriticum	ATCC 10701
Enterobacter cloacae	ATCC 13047
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 35218
Escherichia coli	Wild type[c]
Haemophilus influenzae	ATCC 10211
Klebsiella pneumoniae	ATCC BAA-1705
Micrococcus luteus	ATCC 49732
Neisseria lactamica	ATCC 23970
Proteus vulgaris	ATCC 49132
Pseudomonas aeruginosa	ATCC 10145
Serratia marcescens	Wild type[c]
Staphylococcus aureus	ATCC 25923
Staphylococcus aureus	Wild type[c]
Staphylococcus epidermidis	ATCC 14990
Streptococcus pneumoniae	ATCC 6305
Streptococcus pyogenes	ATCC 12384
Yersinia pestis[a]	ATCC 19428, NR-18[b]
Wolbachia persica	ATCC VR-331

[a] Received through the submission of APHIS/CDC form 2, request to transfer select agents and toxins.

[b] Strain designation from the NIH Biodefense and Emerging Infections Research Resources Repository (BEI Resources).

[c] Wild-type strain from the University of Nebraska Medical Center stock collection.

[d] Previously known at *Bacillus subtilis* var. *niger*.

[e] Abbreviations: LVS, live vaccine strain; ATCC, American Type Culture Collection.

PCR amplifications. The continuous region 10 (CR10) primer set consisted of the oligonucleotides 5′-GCTGATGATTCTAGCCTTAAAGAAG-3′ (CR10 C), 5′-GCCAATCCTACATTTATAGAACCTG-3′ (CR10 S), and 5′-GCCTGGCA TAATTACTGTTTTAGC-3′ (CR10 L). The CR16 primer set was comprised of the primers 5′-CTTGTCAGAGTTGGAGTGAAGC-3′ (CR16 C), 5′-CCACT ACCTCGAATCTTACACAAAG-3′ (CR16 S), and 5′-GTCAAATCACTTTC CTCTCGTTC-3′ (CR16 L). PCR amplifications were performed by using the indicated CR primers and Platinum DNA polymerase (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The cycling parameters were 1 cycle for 2.5 min at 95°C and 40 cycles for 30 s at 95°C, 1 min at 60°C, and 3 min at 72°C, followed by 1 cycle for 5 min at 72°C. PCR products were resolved in a 0.7% (wt/vol) agarose gel, stained with ethidium bromide, and visualized with UV light. Representative amplicons were cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) and sequenced by using M13 forward and reverse primers by the University of Nebraska Medical Center DNA Sequencing Facility.

RESULTS

PCR and chromosomal mapping of amplicons derived from control strains. Computational analysis of the different continuous regions (CRs) previously described (8) suggested that two loci, namely, CR10 and CR16, could be used to differen-

tiate between *F. tularensis* type A and type B strains. The CR10 and CR16 primers were based on chromosomal content within these two loci that was common (C) to both SCHU S4 and LVS or specific to either SCHU S4 (S) or LVS (L). The location of the CR primers and the predicted amplicon size were initially determined by *in silico* analysis for all *Francisella* species and subspecies in which the genome sequence was available, including *F. tularensis* subsp. *mediasiatica*. PCR evaluations for each oligonucleotide pair in the CR10 and CR16 primer sets (C, S, and L) were then performed by using three well-characterized and sequenced strains of *F. tularensis*, specifically SCHU S4 (GenBank accession no. AJ749949; subtype A.I), WY96-3418 (here referred to as WY96) (GenBank accession no. CP000608; subtype A.II), and LVS (GenBank accession no. AM233362; type B).

Preliminary PCR assessments revealed that primer pair CR10 C and CR10 S and primer pair CR16 C and CR16 S were specific for SCHU S4, generating 1.2-kb and 2.3-kb PCR products, respectively. Conversely, primer pair CR10 C and CR10 L and primer pair CR16 C and CR16 L amplified a single PCR product that differed in size for WY96 (1.3 kb and 1.7 kb, respectively) and LVS (2.1 kb and 0.8 kb, respectively). The amplicon sizes obtained with the CR10 and CR16 primers for these three reference strains correlated with the expected lengths based on the nucleotide sequences deposited in the GenBank database (Fig. 1). These results confirmed the *in silico* predictions for all *F. tularensis* subspecies, except *F. tularensis* subsp. *mediasiatica*, for which no DNA was available.

The PCR products obtained for SCHU S4 with primer pair CR10 C and CR10 S and for WY96 and LVS with primer pair CR10 C and CR10 L contained the IS element IS*Ftu1* (Fig. 1A). However, an approximately 300-bp region of the IS*Ftu1* gene in SCHU S4 was apparently deleted, unlike the presumably intact IS*Ftu1* in WY96 and LVS. The absence of *deoD* in the SCHU S4 CR10-derived amplicon, the truncated IS*Ftu1* gene, and the nucleotide differences in primers CR10 S and CR10 L indicated that a unique SCHU S4 chromosomal region was amplified compared to the PCR products obtained with WY96 and LVS. Furthermore, an additional IS element, specifically, IS*Ftu2*, was present in the amplicon derived from LVS with CR10 C and CR10 L but absent in the related WY96 PCR product. These findings verify that this chromosomal region differs between subtype A.II and type B strains even when the same CR10 primers are utilized (Fig. 1A).

For the amplicons derived from primer pairs CR16 C and CR16 S as well as CR16 C and CR16 L, IS*Ftu1* was present in SCHU S4 and WY96, respectively, but absent in LVS (Fig. 1B). The smaller PCR product obtained for LVS with primers CR16 C and CR16 L reflects the absence of this IS element. When the CR16-derived chromosomal regions in SCHU S4 and WY96 were compared, IS*Ftu1* and the adjacent *fnuDI* gene were inverted, with an apparent duplication of *fnuDI* in the latter strain. Since LVS is an attenuated strain, the chromosomal contents of the amplified CR10 and CR16 regions were examined in all available *F. tularensis* subsp. *holarctica* genomes. These assessments indicated that the targeted regions shared considerable nucleotide sequence identity in type B isolates. Collectively, the chromosomal mapping of the CR10 and CR16 primers in the three control strains concurs with the amplicon lengths obtained and confirms that the amplified regions differ in contents among the *F. tularensis* subtype A.I, subtype A.II, and type B strains.

PFGE genotyping. PFGE analysis was shown previously to type and subtype *F. tularensis* (12, 31) and was used as the reference methodology to evaluate a collection of 88 wild-type and 4 well-characterized *F. tularensis* isolates. PFGE was performed by using two different restriction endonucleases (PmeI and BamHI). In these PFGE analyses, *F. tularensis* strains SCHU S4, WY96, and LVS were used as subtype A.I, subtype A.II, and type B controls, respectively. Overall, PFGE analyses of PmeI-digested (Fig. 2) and BamHI-digested (Fig. 3) *F. tularensis* chromosomal DNA were in agreement, identifying 56 type A.I, 15 type A.II, and 21 type B isolates. PFGE using PmeI segregated the subtype A.I isolates largely into two groups, clades A.I.a and A.I.b, as was previously defined by Kugeler and associates (18). However, PFGE analysis utilizing BamHI did not group the subtype A.I isolates into the same clade A.I.a and A.I.b subgroups. A detailed analysis of the clustering patterns obtained with PmeI and BamHI identified five and six subpopulations in the subtype A.I strains, respectively. The PFGE patterns of the 15 subtype A.II isolates were more divergent than those of the subtype A.I strains, as previously reported (18). PFGE analysis with both PmeI and BamHI indicated the presence of six discernible subtype A.II groups, whereas all 21 type B isolates were indistinguishable. For *F. tularensis* subsp. *novicida* and *F. philomiragia*, PFGE patterns obtained with PmeI and BamHI indicated that they were both outliers and distantly related to *F. tularensis* type A and type B strains (data not shown). Together, these data validate the PFGE patterns obtained with the use of either PmeI or BamHI to identify the *F. tularensis* subspecies or subtype.

Subspecies identification and subtyping by PCR. The CR10 and CR16 primers were utilized in a PCR to type a collection of wild-type *F. tularensis* isolates. Typing results obtained with the CR-derived amplicons were compared to results of PFGE genotyping with PmeI and BamHI. These results are summarized in Table 2. The CR-derived amplification products differentiated the *F. tularensis* strains into types and subtypes that corroborated the results of genotyping by PFGE.

The majority of subtype A.I isolates analyzed with primers CR10 C and CR10 S generated PCR products that were 1.2 kb in size (43 strains). An amplicon of this length was also observed for the subtype A.I prototype strain, SCHU S4. The remainder of the subtype A.I isolates (14 strains) produced a 1.5-kb amplicon, indicating that this locus differed by approximately 300 bp from the other subtype A.I strains (Table 2). Primer pair CR10 C and CR10 L produced a 1.3-kb amplicon for all of the subtype A.II isolates and a 2.1-kb amplicon for all of the type B strains (Table 2). For *F. tularensis* subsp. *novicida*, a 0.4-kb PCR product was produced with primers CR10 C and CR10 L, and no amplicon was observed with primer pair CR10 C and CR10 S.

The utilization of primers CR16 C and CR16 S produced either a 2.3-kb (54 strains) or a 2.0-kb (3 strains) amplicon for the *F. tularensis* subtype A.I isolates, with the larger PCR product being amplified from the SCHU S4 genome (Table 2). For the subtype A.II strains, primers CR16 C and CR16 L produced either a 1.7-kb or a 1.4-kb PCR product, with the generation of the larger 1.7-kb amplicon from WY96 (Table

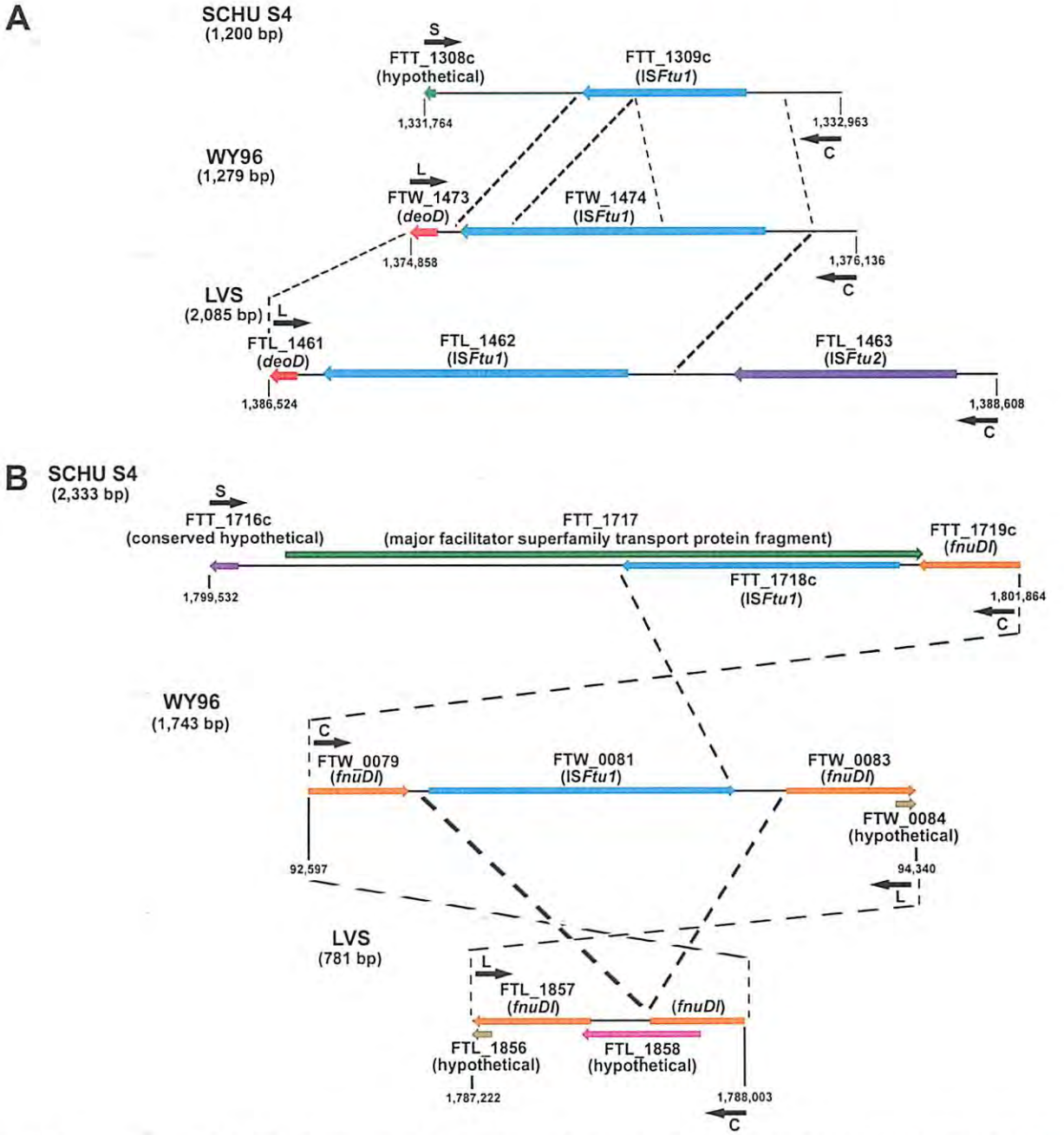


FIG. 1. Chromosomal map of CR10 and CR16 primer pair locations in *F. tularensis* subsp. *tularensis* subtypes A.I and A.II and *F. tularensis* subsp. *holarctica* type B. The chromosomal regions amplified with the CR10 (A) and CR16 (B) primer sets from the sequenced reference *F. tularensis* strains are shown. SCHU S4 (GenBank accession no. AJ749949), WY96-3418 (WY96) (GenBank accession no. CP000608), and LVS (GenBank accession no. AM233362) represent subtype A.I, subtype A.II, and type B strains, respectively. The resulting amplicon size is indicated below the appropriate strain in parentheses. The forward and reverse CR (C, S, or L) primers are denoted by black arrows. The corresponding genes in the various strains for each panel are indicated by the same color with the locus identifier number. The beginning and ending nucleotide positions for the mapped loci are denoted below the respective chromosomal regions. Dashed lines designate relative locations and organizations of the corresponding nucleotide sequences in the *F. tularensis* genomes.

2). All type B isolates produced a 0.8-kb amplicon with primers CR16 C and CR16 L (Table 2). No amplicons were obtained with either primer pair CR16 C and CR16 S or CR16 C and CR16 L for *F. tularensis* subsp. *novicida*.

Overall, these results demonstrated that the same PCR product profile was generated with all four CR primer pairs for both the reference and wild-type strains of the same subtype and/or type. To reduce the number of PCR samples and further test the capability of the DISA assay, the CR10 and CR16 primer pairs were reduced to only two sets of primers, with each set containing the three associated primers (CR10 C, CR10 S, and CR10 L as well as CR16 C, CR16 S, and CR16 L).

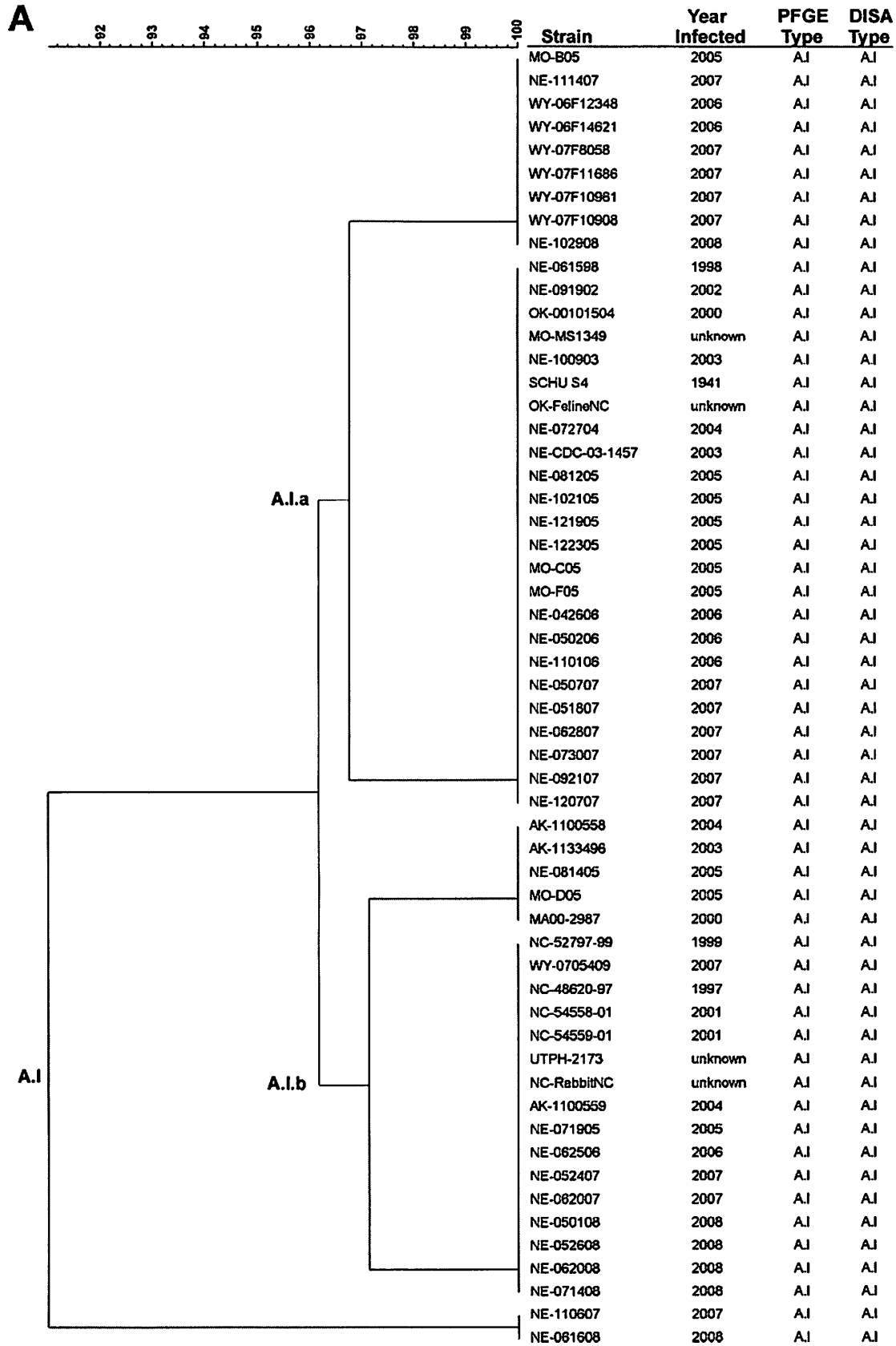
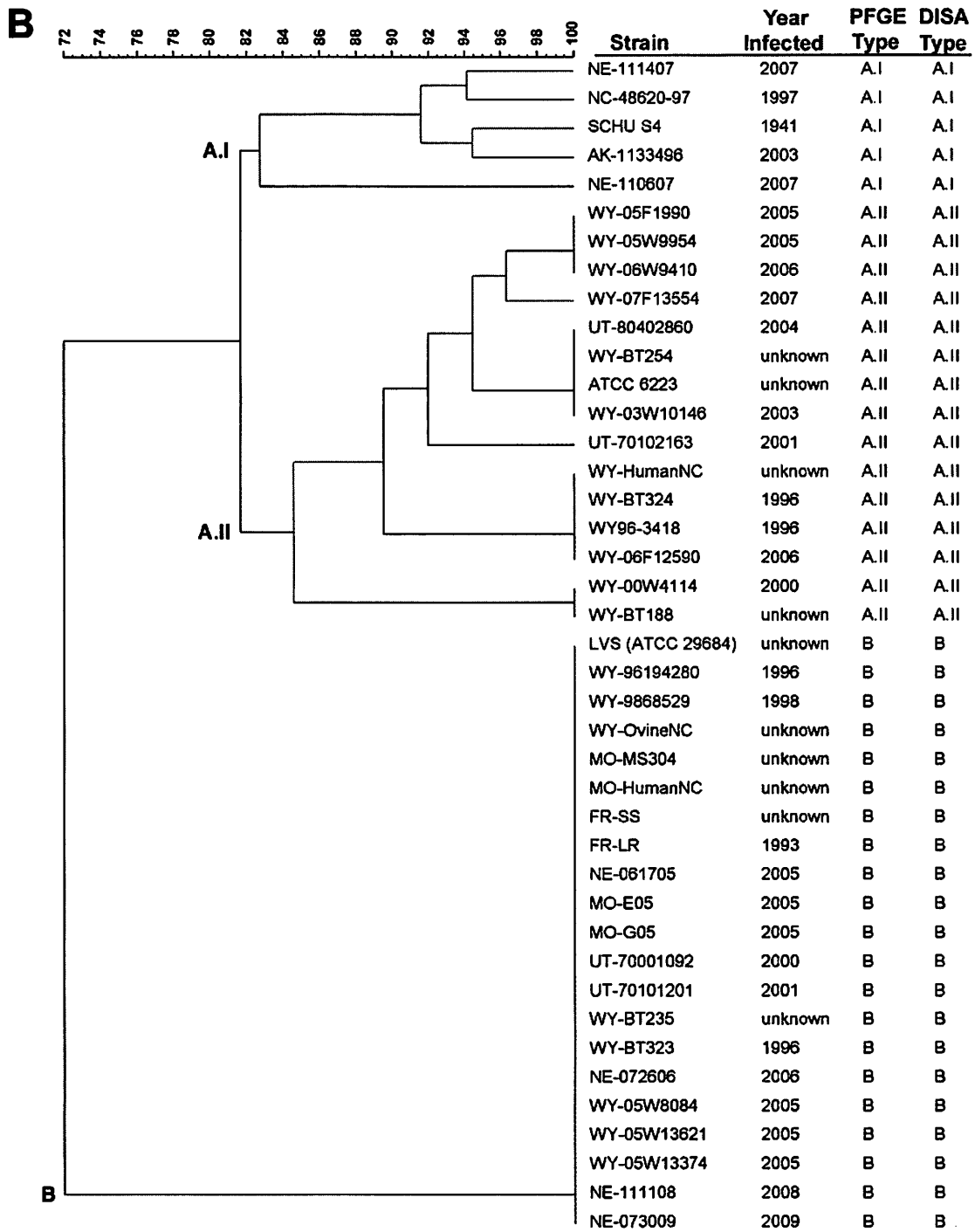


FIG. 2. Dendrogram of PFGE patterns obtained with PmeI-digested *F. tularensis* subsp. *tularensis* subtype A.I and the apparent clade A.I.a and A.I.b subpopulations (A) as well as *F. tularensis* subsp. *tularensis* subtype A.II and *F. tularensis* subsp. *holarctica* type B (B). A representative PmeI-digested *F. tularensis* A.I strain from each of the five clusters is shown in the upper part of B for comparison. Distance is displayed in relative units.



Strain	Year Infected	PFGE Type	DISA Type
NE-111407	2007	A.I	A.I
NC-48620-97	1997	A.I	A.I
SCHU S4	1941	A.I	A.I
AK-1133496	2003	A.I	A.I
NE-110607	2007	A.I	A.I
WY-05F1990	2005	A.II	A.II
WY-05W9954	2005	A.II	A.II
WY-06W9410	2006	A.II	A.II
WY-07F13554	2007	A.II	A.II
UT-80402860	2004	A.II	A.II
WY-BT254	unknown	A.II	A.II
ATCC 6223	unknown	A.II	A.II
WY-03W10146	2003	A.II	A.II
UT-70102163	2001	A.II	A.II
WY-HumanNC	unknown	A.II	A.II
WY-BT324	1996	A.II	A.II
WY96-3418	1996	A.II	A.II
WY-06F12590	2006	A.II	A.II
WY-00W4114	2000	A.II	A.II
WY-BT188	unknown	A.II	A.II
LVS (ATCC 29684)	unknown	B	B
WY-96194280	1996	B	B
WY-9868529	1998	B	B
WY-OvineNC	unknown	B	B
MO-MS304	unknown	B	B
MO-HumanNC	unknown	B	B
FR-SS	unknown	B	B
FR-LR	1993	B	B
NE-061705	2005	B	B
MO-E05	2005	B	B
MO-G05	2005	B	B
UT-70001092	2000	B	B
UT-70101201	2001	B	B
WY-BT235	unknown	B	B
WY-BT323	1996	B	B
NE-072606	2006	B	B
WY-05W8084	2005	B	B
WY-05W13621	2005	B	B
WY-05W13374	2005	B	B
NE-111108	2008	B	B
NE-073009	2009	B	B

FIG. 2—*Continued.*

These tests produced the same PCR product profiles as those of the four primer pairs but with half as many reactions.

Amplification specificity and sensitivity of CR primers. The CR primer specificity for *F. tularensis* was evaluated by using an exclusivity panel (n = 24) (Table 1) that included chromosomal DNA from the near neighbors *F. philomiragia* and *Wolbachia persica*. Neither the CR10 nor CR16 primer pairs resulted in any prominent PCR products for these bacterial samples, with the exception of *W. persica*, in which a 250-bp amplicon was generated by use of primers CR10 C and CR10 L.

The applicability of PCR amplification with the CR primers in environmental studies to detect the presence of *F. tularensis* was examined by using arthropods, pond water, and soil. Fifty-four ticks, including *Dermacentor andersoni*, *Dermacentor variabilis*, and *Amblyomma americanum*, species known to

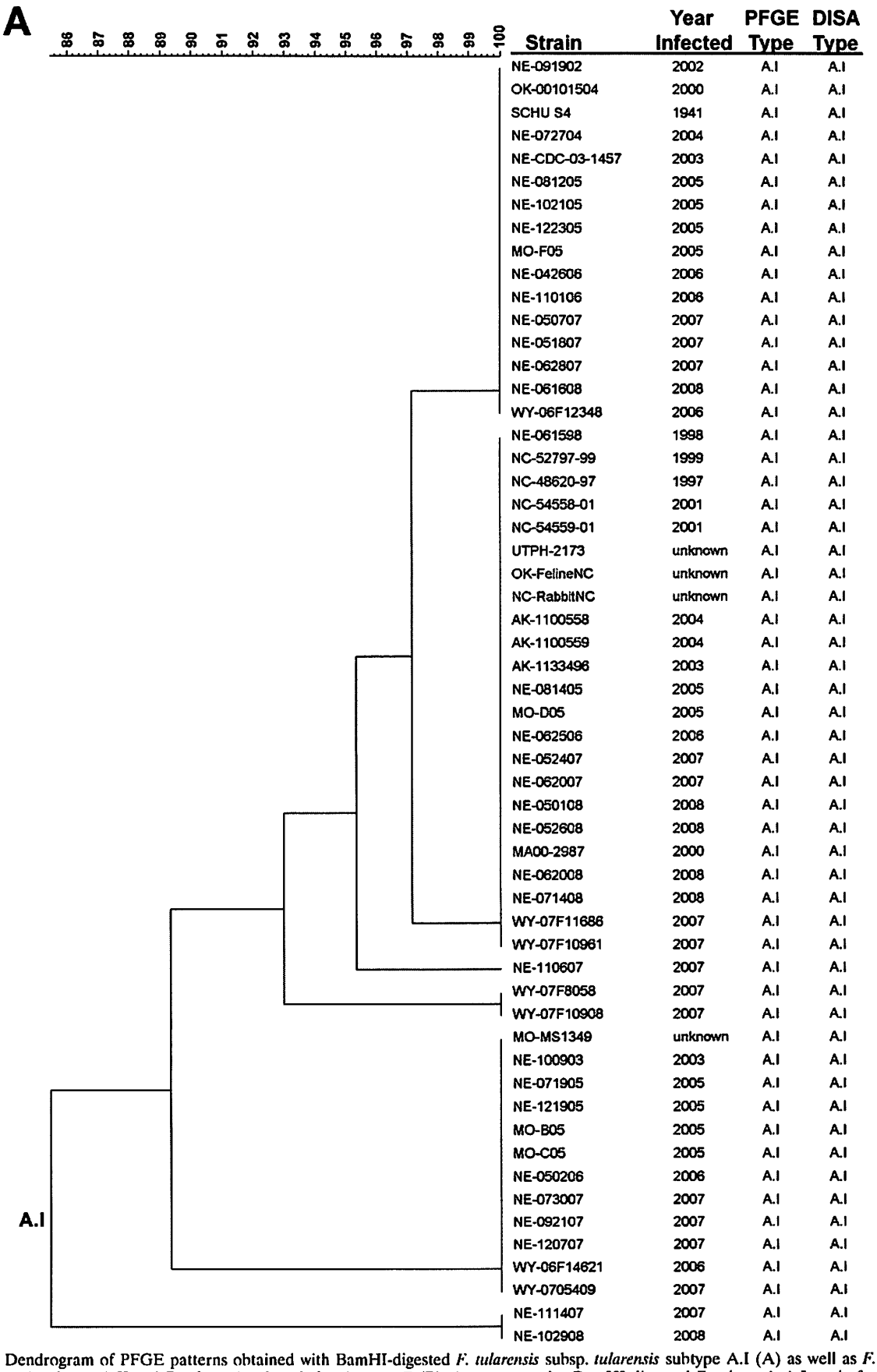
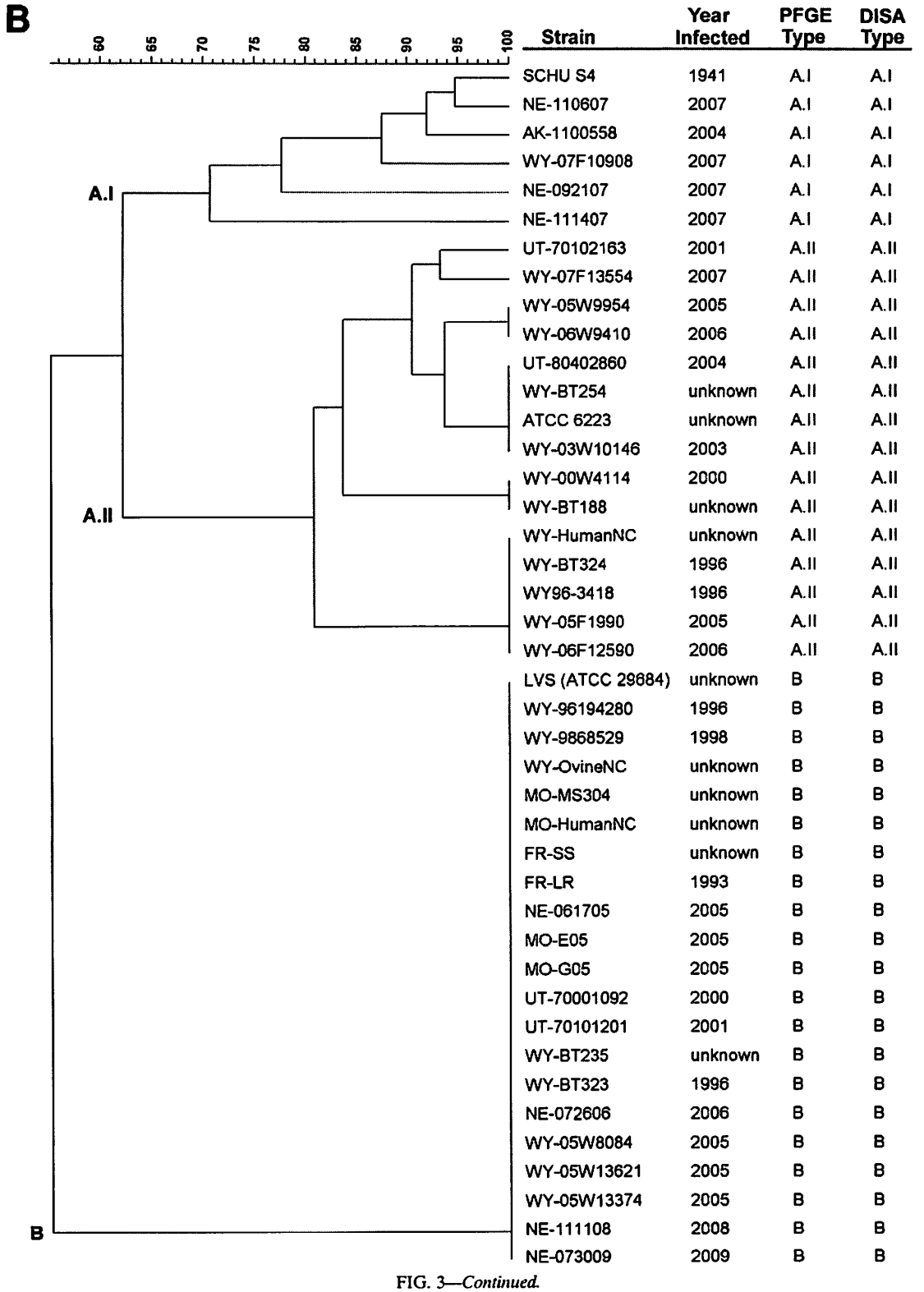


FIG. 3. Dendrogram of PFGE patterns obtained with BamHI-digested *F. tularensis* subsp. *tularensis* subtype A.I (A) as well as *F. tularensis* subsp. *tularensis* subtype A.II and *F. tularensis* subsp. *holarctica* type B (B). A representative BamHI-digested *F. tularensis* A.I strain from each of the six clusters is shown in the upper part of B for comparison. Distance is displayed in relative units.



Strain	Year Infected	PFGE Type	DISA Type
SCHU S4	1941	A.I	A.I
NE-110607	2007	A.I	A.I
AK-1100558	2004	A.I	A.I
WY-07F10908	2007	A.I	A.I
NE-092107	2007	A.I	A.I
NE-111407	2007	A.I	A.I
UT-70102163	2001	A.II	A.II
WY-07F13554	2007	A.II	A.II
WY-05W9954	2005	A.II	A.II
WY-06W9410	2006	A.II	A.II
UT-80402860	2004	A.II	A.II
WY-BT254	unknown	A.II	A.II
ATCC 6223	unknown	A.II	A.II
WY-03W10146	2003	A.II	A.II
WY-00W4114	2000	A.II	A.II
WY-BT188	unknown	A.II	A.II
WY-HumanNC	unknown	A.II	A.II
WY-BT324	1996	A.II	A.II
WY96-3418	1996	A.II	A.II
WY-05F1990	2005	A.II	A.II
WY-06F12590	2006	A.II	A.II
LVS (ATCC 29684)	unknown	B	B
WY-96194280	1996	B	B
WY-9868529	1998	B	B
WY-OvineNC	unknown	B	B
MO-MS304	unknown	B	B
MO-HumanNC	unknown	B	B
FR-SS	unknown	B	B
FR-LR	1993	B	B
NE-061705	2005	B	B
MO-E05	2005	B	B
MO-G05	2005	B	B
UT-70001092	2000	B	B
UT-70101201	2001	B	B
WY-BT235	unknown	B	B
WY-BT323	1996	B	B
NE-072606	2006	B	B
WY-05W8084	2005	B	B
WY-05W13621	2005	B	B
WY-05W13374	2005	B	B
NE-111108	2008	B	B
NE-073009	2009	B	B

FIG. 3—*Continued.*

transmit *F. tularensis* in North America (26), were assessed for the presence of *F. tularensis*.

PCR with the CR primers revealed that one *D. andersoni* tick and one *D. variabilis* tick produced products consistent with the presence of *F. tularensis*, whereas the remainder of the ticks did not produce any amplicons. Importantly, the PCR product sizes obtained with the CR primers provided subspecies and subtype details. Primer pair CR10 C and CR10 L and

TABLE 2. Comparison of typing results obtained by PCR with the CR primers and by PFGE with PmeI or BamHI for *F. tularensis* subsp. *tularensis* (subtypes A.I and A.II), *F. tularensis* subsp. *holarctica* (type B), and *F. tularensis* subsp. *novicida* isolates

No. of *F. tularensis* isolates	CR primer pair	Amplicon size (kb)	Type determined by:		
			PCR-based typing with CR primers	PFGE-based typing with PmeI	PFGE-based typing with BamHI
43[a]	CR10 C + CR10 S	1.2	A.I	A.I	A.I
14	CR10 C + CR10 S	1.5	A.I	A.I	A.I
54[a]	CR16 C + CR16 S	2.3	A.I	A.I	A.I
3	CR16 C + CR16 S	2.0	A.I	A.I	A.I
15[b]	CR10 C + CR10 L	1.3	A.II	A.II	A.II
14[b]	CR16 C + CR16 L	1.7	A.II	A.II	A.II
1	CR16 C + CR16 L	1.4	A.II	A.II	A.II
21[c]	CR10 C + CR10 L	2.1	B	B	B
21[c]	CR16 C + CR16 L	0.8	B	B	B
1[d]	CR10 C + CR10 L	0.4	*F. tularensis* subsp. *novicida*	*F. tularensis* subsp. *novicida*	*F. tularensis* subsp. *novicida*
1[d]	CR10 C + CR10 S				
1[d]	CR16 C + CR16 L				
1[d]	CR16 C + CR16 S				

[a] Includes *F. tularensis* subsp. *tularensis* strain SCHU S4 (subtype A.I).
[b] Includes *F. tularensis* subsp. *tularensis* strain WY96-3418 (subtype A.II).
[c] Includes *F. tularensis* subsp. *holarctica* LVS (type B).
[d] *F. tularensis* subsp. *novicida* Utah 112 (ATCC 15482).

primer pair CR16 C and CR16 L produced amplicons indicating the presence of *F. tularensis* subsp. *tularensis* subtype A.II in the *D. andersoni* tick. Amplification products derived with primer pair CR10 C and CR10 L and primer pair CR16 C and CR16 L and DNA isolated from the *D. variabilis* tick were characteristic of *F. tularensis* subsp. *holarctica* (type B). The amplicons derived from these two ticks were sequenced, and the data corresponded to the appropriate *F. tularensis* subtype and/or type as determined by the DISA assay. Results from two commercially available PCR-based test kits (Idaho Technology, Inc., Salt Lake City, UT) that detect *F. tularensis* were also positive using DNA extracts from these two ticks. However, the commercial assays could not type the *F. tularensis* isolates. No viable *F. tularensis* was recovered from these two tick specimens by culturing on chocolate agar plates, and no other environmental samples were positive with either the commercial or CR primer pairs. Together, these data demonstrated that the CR primers were specific for *F. tularensis* and capable of detection and subspecies or subtype identification for both the clinical and environmental isolates examined.

As a control to verify the presence of *Francisella*-like endosymbionts in the ticks, PCR amplifications were performed by using primers known to amplify target genes in *Francisella*-like bacteria (3, 11, 24, 30). The *Francisella*-like endosymbiont *W. persica* served as a positive control. In addition, commercially available test kits (Idaho Technology, Inc.) that detect only *F. tularensis* subspecies were utilized in these assessments. Ticks that produced PCR products with the primers designed to amplify *Francisella*-like bacteria were further evaluated with the commercial *F. tularensis*-specific assays as well as with the DISA assay. If no amplicons were produced with the *F. tularensis*-specific assays but PCR products were produced with the primers for *Francisella*-like bacteria, the respective amplicons were sequenced. The subsequent nucleotide sequences were compared to those deposited in the databases, confirming the presence of *Francisella*-like endosymbionts and the absence of *F. tularensis* or *F. philomiragia* in the associated ticks. These findings demonstrated that the CR primers did not amplify DNA from *Francisella*-like endosymbionts known to exist in ticks.

The limit of detection for the PCR amplification of *F. tularensis* with the CR primers under standard PCR conditions described in Materials and Methods was determined to be approximately 20 copies of the 1.9-Mbp *F. tularensis* chromosome. Therefore, PCR with the CR primers provided a detection of *F. tularensis* at a level naturally present in environmental sources and achieved an identification of the subspecies or subtype.

Sequence analysis of amplicons. Representative PCR products obtained with the CR primers for each size group were cloned and sequenced to determine content. The DNA sequences of the PCR products generated with the CR10 and CR16 primers for these strains were in agreement with the expected lengths (Table 2) and mapped loci (Fig. 1). The size difference observed for the 1.2-kb and 1.5-kb amplicons in the *F. tularensis* subtype A.I clade obtained with primers CR10 C and CR10 S was due to a 312-bp deletion in the smaller PCR product (Table 3).

With primer pair CR16 C and CR16 S, either a 2.3-kb or a 2.0-kb amplicon was generated for the subtype A.I strains, while primers CR16 C and CR16 L resulted in either a 1.7-kb or a 1.4-kb PCR product for the subtype A.II isolates. In both cases, the smaller amplification product was again due to a 312-bp deletion (Table 4). The 312-bp deleted region in the smaller CR10- and CR16-derived amplicons correlated with the middle region of the IS*Ftu1* transposase gene. Single-nucleotide polymorphisms (SNPs) were also noted for some of the CR-derived PCR products, as indicated in Tables 3 and 4.

DISCUSSION

As with other virulent pathogens, such as *Bacillus anthracis* and *Yersinia pestis*, *F. tularensis* is highly monomorphic, with greater than 99% sequence similarity between type A

TABLE 3. Sequence analysis of representative amplicons obtained by PCR with the CR10 primer set relative to *F. tularensis* SCHU S4, WY96-3418, or LVS[a]

F. tularensis isolate	Type	CR10 primer pair	Amplicon size (kb)	Insertion	SNP[b]
SCHU S4	A.I	C + S	1.2		
NE-061598	A.I	C + S	1.2		
NC-52797-99	A.I	C + S	1.2		nt 896/A→T; nt 955/T→C
NE-071905	A.I	C + S	1.5	312 bp at nt 539-850[c]	nt 512/T→C
WY-0705409	A.I	C + S	1.5	312 bp at nt 539-850[c]	nt 1135/A→G; nt 1244/T→C
WY96-3418	A.II	C + L	1.3		
WY-00W4114	A.II	C + L	1.3		nt 755/T→C
WY-HumanNC	A.II	C + L	1.3		
LVS	B	C + L	2.1		

[a] Nucleotide (nt) sequences for reference strain SCHU S4, WY96-3418, and LVS amplicons were obtained from GenBank accession no. AJ749949, CP000608, and AM233362, respectively.
[b] SNP, single-nucleotide polymorphism.
[c] Derivative of IS*Ftu1*.

and type B strains (27, 28). Genetically monomorphic pathogens may have recently passed through a population bottleneck, reducing or abolishing genetic diversity (1). Despite the considerable conservation of overall genomic content among *F. tularensis* subspecies, the organizations of discrete genetic elements differ (8). The observed translocations and rearrangements appear to be mediated by the movement of IS elements, most notably by the highly abundant IS*Ftu1* gene (27). Seven different types of IS elements have been found within SCHU S4 and the closely related subtype A.I strain NE-061598, including IS*Ftu1* (50 copies); IS*Ftu2* (16 copies); IS*Ftu3* and IS*Ftu6* (3 copies each); and IS*Ftu4*, IS*Ftu5*, and IS*Sod13* (1 copy each) (23). IS elements appear to be the primary driving force for the evolution of the *Francisella* chromosome, contributing to genome decay and subsequent intracellular dependence.

Although there are similar numbers of IS*Ftu1* genes in *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) and many more copies of IS*Ftu2* in type B than in type A strains, genomic rearrangements in type B strains are much less frequent (32). The low level of genetic diversity and high frequency of transposable elements in the type B clade support the hypothesis that *F. tularensis* subsp. *holarctica* emerged after *F. tularensis* subsp. *tularensis* and, therefore, is more clonal. In addition, the wider geographic dispersion of *F. tularensis* subsp. *holarctica* supports the correlation between this pathogen either having a higher capacity to survive in different environmental niches than *F. tularensis* subsp. *tularensis* or causing less morbidity in the associated host. The availability of a rapid PCR-based method for examining additional wild-type isolates may provide new insights into the mechanism for the observed geographic associations between these two subspecies.

Despite the subtype A.I and A.II strains having similar numbers of IS elements (6, 20), there is considerable overall variation in chromosome architectures (18, 23). The current study revealed that a 312-bp insertion or deletion derived from IS*Ftu1* was responsible for the size heterogeneity within the CR-derived amplicons in the subtype A.I and A.II strains but that discrimination between these two clades was still apparent. In *Mycobacterium*, *Salmonella*, and other pathogens the locations of IS elements have been sufficiently stable for use as markers in restriction fragment length polymorphism studies and typing for epidemiological purposes (21). In *Francisella*, the spatial location of IS elements and their involvement in the plasticity of the genome can

TABLE 4. Sequence analysis of representative amplicons obtained by PCR with the CR16 primer set relative to *F. tularensis* SCHU S4, WY96-3418, or LVS[a]

F. tularensis isolate	Type	CR16 primer pair	Amplicon size (kb)	Insertion	Deletion	SNP[c]
SCHU S4	A.I	C + S	2.3			
NE-061598	A.I	C + S	2.3			
WY-07F10908	A.I	C + S	2.0	T at nt 1193	312 bp at nt 1282-1593[b]	nt 1202/T→C
NE-052608	A.I	C + S	2.0		312 bp at nt 1282-1593[b]	
WY-07F8058	A.I	C + S	2.0	T at nt 1193	312 bp at nt 1282-1593[b]	nt 1202/T→C
WY96-3418	A.II	C + L	1.7			
WY-00W4114	A.II	C + L	1.7			
WY-HumanNC	A.II	C + L	1.7			
WY-07F13554	A.II	C + L	1.4		312 bp at nt 691-1002[b]	nt 567/C→T; nt 643/A→G; nt 1126/G→C; nt 1228/T→C; nt 1389/T→C; nt 1650/T→C
LVS	B	C + L	0.8			
WY-05W8084	B	C + L	0.8			
UT-70101201	B	C + L	0.8			nt 290/A→T

[a] Nucleotide sequences for reference strain SCHU S4, WY96-3418, and LVS amplicons were obtained from GenBank accession no. AJ749949, CP000608, and AM233362, respectively.
[b] Derivative of IS*Ftu1*.
[c] SNP, single-nucleotide polymorphism.

also be exploited. Our future studies will determine whether the DISA method will provide a means to detect and monitor the emergence of new substrains of this highly infectious pathogen.

Type A.I, A.II, and B strains of *F. tularensis* are present in North America, with hard ticks being considered the major biological vector of tularemia in this region (26). Since *F. tularensis* survival is sustained throughout the tick's life cycle from larva to nymph to adult, and since tick morphogenesis requires a blood meal, pathogen transmission to a new host may occur at each stage. Therefore, a single infected tick may be responsible for establishing a new or recurring focus of disease in the environment. The multidirectional nature of zoonosis, the presence of *Francisella*-like endosymbionts in ticks, and the broad host range of *F. tularensis* have complicated epidemiological investigations into diseases caused by this pathogen. A field-deployable assay would be useful for the characterization of infection outbreaks and the identification of their point source or location. The PCR-based DISA method targeting IS elements provides a reliable approach for the rapid evaluation of ticks, animals, and humans potentially infected with *F. tularensis*. Furthermore, an accurate surveillance of *F. tularensis* vectors and reservoirs will provide a better understanding of this pathogen's biology.

Molecular-based assays for the detection of infectious diseases have continued to evolve in order to address the problem of false-positive results due to the cross-hybridization of primers with unrelated sequences with unexpected homology. Some of the most effective changes have been the incorporation of multiple targets into the assay, with the criterion for positivity being the amplification of more than one target. Although several real-time PCR assays have been developed to differentiate isolates of *F. tularensis* that are of types A and B (19) or types A.I and A.II (22), a more comprehensive method was needed to simultaneously and accurately genotype this species. The DISA assay offers this capability and requires only two sets of primers, unlike typing by PCR-based multilocus variable-number tandem repeat analysis. Furthermore, the DISA method takes advantage of the presence of multiple inherent targets within *Francisella*, and this approach will be used for the future development of a real-time molecular assay.

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